NONSELECTIVE ENRICHMENT FOR ADENINE MUTANTS BY FLOW CYTOMETRY: A NEW ANALYTICAL APPROACH TO EUKARYOTIC DNA ALTERATIONS

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ABSTRACT

The expression of certain adenine biosynthetic mutants in the yeast Saccharomyces cerevisiae results in a red colony color. This phenomenon has historically provided an ideal genetic marker for the study of mutation, recombination, and aneuploidy in lower eukaryotes by classical genetic analysis. In this paper, it is reported that cells carrying ade1 and/or ade2 mutations exhibit primary fluorescence. Based on this observation, the nonselective enrichment of yeast cultures for adenine mutants using the fluorescence activated cell sorter has been achieved. The advantages of this approach over conventional genetic analysis of mutation, recombination, and mitotic chromosomal stability include speed and accuracy in acquiring data for large numbers of clones. Using appropriate strains, the cell sorter has been used for the isolation of both forward mutations and chromosomal loss events in S. cerevisiae. The resolution power of this system and its non-invasiveness can easily be extended to more complex organisms, including mammalian cells, in which analagous metabolic mutants are available.

The analysis of changes in cellular genomic DNA arrangement is of primary importance to the study of chromosomal dynamics and the elucidation of predisposing factors in genetic disease and neoplasia. Recently, the use of the fluorescence activated cell sorter (FACS) has allowed the rapid and reproducible detection of karyotypic abnormalities in clones of malignant cells, following staining of isolated chromosomes with DNA-specific fluorochromes (1). However, because the changes in genomic DNA content are relatively small, the detection of individual cells with chromosomal aberrations in viable populations has not been successful using these dyes. Nonetheless, other genetic phenomena, such as lacZ plasmid replication and segregation in S. cerevisiae have been studied using FACS detection of β-galactosidase activity with a fluorogenic substrate and trapping reagent (2). This method, however, has the significant drawback of destroying cell viability.

In this paper we describe a generally applicable approach for flow cytometry analysis of chromosomal segregation in S. cerevisiae, which allows for the highly efficient sorting and recovery of viable mutants. The mutant clones can subsequently be cultured and analyzed for specific recombinational and segregational events. The system is based on the well-known 'Roman effect' colony color assays (3). In the 'Roman effect', a cell-limited red pigment accumulates in haploid cells carrying mutations in the ADE1 and/or ADE2 genes (or in diploid cells homozygous for either or both of these mutations) which allows for the visual identification of mutant clones by colony color.

Mutations in several other genes of the adenine biosynthetic pathway are epistatic to these mutations, such that ade1 ade6 double mutant strains, for example, are white. In addition, the loss of the wild type alleles at heterozygous ade1/ADE1 (chromosome I) and/or ade2/ADE2 (chromosome XV) loci in diploids leads to accumulation of red pigment and a change in colony color from white to red. Since we found that the cells from red colonies were fluorescent, we used the FACS to rapidly enumerate and sort very large populations of red and white yeast cells. In fact, mutant populations could be enriched by greater than 10,000 fold without applying selective pressure.

As an illustration of the resolution power of the cell sorter, the following example can be considered. A single yeast colony may contain as many as 5×10^7 colony forming units (CFU's). Using conventional genetic analysis without selection, it would be necessary to plate these cells onto 50,000 plates (at 1000 CFU's per plate) in order to identify all the possible red chromosomal loss mutants present in the colony. At a flow rate of 5,000 cells per second, the FACS can accomplish essentially the same task in less than three hours.

Fluorescence properties of red pigmented yeast cells.

Fluorescence microscopy of cells from the red strain CBN1 is shown in Figures 1a and 1b. Both red and green fluorescence were observed using different filter combinations. The fluorescence appeared to be evenly distributed throughout the cell, except in the case of highly vacuolated cells for which fluorescence was excluded from vacuoles (not shown). White strains (e.g., strain

S288C) not carrying ade1 or ade2 mutations were not visible under these conditions (data not shown). Fluorometry scans of the trichloroacetic acid-insoluble fractions of cytosol preparations of strains CBN1-R (red) and S288C (white) are shown in Figure 1c. Subtraction of the fluorescence emmission profile of strain S288C from that of the strain CBN1 revealed a broad peak in the visible spectrum with a maximum at 569 nm.

FACS analysis of mixed populations.

Artificial mixtures of red and white cells (strain CBN2-W white, and CBN2-R/red) were analyzed using the FACS 440 cell sorter with excitation at 488 nm. In Figure 2, three-dimensional isometric graphs of these data are shown. Red fluorescence intensity (F1-axis), green fluorescence intensity (F2-axis), and cell number (Z-axis) were the three parameters analyzed. As expected from Figure 1, cells from the red strain fluoresced in both green and red dimensions. Clearly defined peaks corresponding to red and white populations could be discerned when the minority population in the red/white mixture consisted of as low as 0.5% of the total cells. Similar results were obtained using strains CBN1-W and CBN1-R.

Using the same filters, but with excitation at 514 nm, red and white peaks were still separated, however white cells actually appeared to exhibit more intense green fluorescence than red cells. Analysis of mixtures of red and white cells (CBN1-R and CBN1-W) with excitation at the 514 nm laser line is shown in Figure 3. This apparent green fluorescence of white cells was an

artifact due to the 514 nm (green) laser light passing through the 530 nm band pass filter, as white cells did not exhibit fluorescence in the fluorescence microscope using any of the filter combinations tried. Regardless of this artifact, 514 nm excitation was more efficient than 488 nm excitation for the peak separation and sorting of red and white populations.

The clear peak definition obtained using the FACS analysis of mixtures of red and white yeast cells (Figures 2 and 3), indicated that cells which change color following genetic alterations such as mutation, conversion, or chromosomal loss, could be easily isolated by flow cytometry. In a demonstration of this, sorting windows were defined around red (strain CBN2*R) and white (strain CBN2-W) peaks in the red/green fluorescence intensity plane, and flowing cells representing red and white populations were gated into tubes based on red and green fluorescence. Subsequently, sorted cells were plated onto complete media and scored for the percentages of red and white colonies formed after incubation. Table 1 shows representative data from the experiment illustrating the relative efficiencies of sorting red or white cells out of red/white mixtures. Red cells sorted from mixtures containing 10% red cells/90% white cells were highly homogeneous, forming greater than 90% red colonies. Conversely, white cells sorted from mixtures containing 10% white cells/90% red cells formed about 70% white colonies. Hence, red cells were more efficiently enriched from mixtures than white cells. Also, the homogeneity of sorted cells in terms of the color of the colonies which they formed, was

generally much higher when smaller sorting windows were defined or when several cycles of enrichment by sorting were performed. We estimate that the greatest enrichment of red cells from white populations obtained to date has been 15,000 fold.

Isolation of forward mutants and aneuploids.

The white haploid strain S288C can acquire the red phenotype only by forward mutation in the ADE1 and/or ADE2 genes. In order to demonstrate the capability of the FACS in isolating clones representing rare mutational events, we used the FACS to analyze 3.5×10^6 cells from this strain and isolated the cells appearing in the red window by sorting. Two red colonies, which were adenine auxotrophs but otherwise retained the genotype of S288C, appeared. This corresponds to a forward mutation frequency on the order of 10^{-6}, in good agreement with known values.

The diploid strain CBN2-W was constructed to be homozygous for the temperature sensitive cell division cycle mutation cdc6. At the restrictive temperature this strain undergoes chromosomal loss at high frequencies (4). This phenomenon is currently exploited for the mapping of unknown genetic loci by chromosomal loss. Since CBN2-W is heterozgous at the ade1/ADE1 and ade2/ADE2 loci, this strain provided for a convenient assessment of the ability of the FACS to isolate true monosomic (hypodiploid) mutants. Following induction of chromosomal loss in strain CBN2 by incubation at $36^{\circ}C$ in liquid medium for six hours, cells were analyzed using the FACS. Contour windows were defined as described above and red cells were sorted from the

predominantly white population. In order to increase the efficiency of the enrichment, several cycles of sorting were performed with a 10 to 20 fold enrichment in each cycle. More than 10,000 red colonies could be obtained in a single sorting experiment. A significant proportion of colonies had red/white sectors, indicating that missegregation occurred in the mitotic division immediately preceeding the sorting. The red chromosomal loss mutants are currently being characterized further by complementation analysis using ade1 and ade2 tester strains to determine the relative frequencies of loss of chromosomes I and XV (Bruschi and Chuba, manuscript in preparation).

Pigment accumulation depends upon the age of the cell.

One possible explanation of the fact that red cells were more efficiently enriched from mixed populations than vice versa (Table 1) could be that there exist some cells in the population which have the red genotype, but have not yet accumulated enough of the fluorochrome to be detected as phenotypically red. These cells would be detected by the FACS as white, but form red colonies. Indeed, red colonies in log phase growth often have a white perimeter ('fried egg' appearance) which later disappears. Assuming that individual cells accumulate the fluorescence at the same rate, we hypothesized that these phenotypically white but genotypically red cells should be the younger (smaller cells). In order to test this hypothesis, a red colony from strain CBN1-R with a relatively large white perimeter was analyzed in the FACS (Figure 4). Although this colony was genetically homogeneous, both red and white peaks were evident (in this case the white

predominates). Sorting windows were defined around these peaks, and the cell populations were examined separately for the parameters of forward (cell size) and right-angle (cell shape) light scatter. Figure 4 shows that the phenotypically red cells were clearly larger (older cells), scattering more light in both the forward and right-angle directions, and were more heterogeneous for these two parameters than the white cells. For many applications the presence of a small percentage of cells which have not accumulated sufficient pigment for detection may not present a problem. To minimize this difficulty, however, cells can be grown into mid-stationary phase on complete medium with low adenine (10 μg/ml), or incubated for several days at 4°C to allow accumulation of the pigment. Increasing the concentration of glucose in the medium to 4% also increases pigment accumulation in some strains. Since the pigment does not accumulate during anaerobic growth (5), vigorous aeration is recommended for broth cultures.

We are presently extending the FACS approach to isolation of chromosomal loss mutants using hyperhaploid yeast strains in which loss of disomic chromosomes leads to a change in colony color from white to red, or from red to white (6). Using the FACS/red-white system we should be able to analyze loss events in disomic strains with at least six yeast chromosomes (i.e., chromosomes 1,4,6,7,13, and 15) on which ADE biosynthetic genes are carried. Furthermore, because some of the adenine mutations are suppressible, loss of other chromosomes can theoretically be

studied if appropriate suppressors are present in heterozygous condition, so that loss of a disomic chromosome carrying a suppressor would also lead to a color change. Until now, the analysis of chromosome loss in disomic strains has been limited to a few chromosomes which carry recessive drug resistant markers (6), so that loss of only one chromosome of a pair is detected. In this way however, information concerning the directionality of chromosome loss is forfeited. The FACS system will determine the directionality of chromosomal loss if different ADE biosynthetic mutations are located in trans on either chromosome of a pair. Once heteroallelic markers in ADE1 and ADE2 genes are constructed (in progress), analysis of the polarity of gene conversion of the wild type or the mutant alleles (7), using the FACS will also be facilitated.

Some other significant applications come to mind. For example using the shuttle vector plasmid YRp16, which carries the ADE8 gene, it is possible to enrich for recombinants generated by insertional inactivation in ADE8 using the FACS. One attractive feature of the system described here is the theoretical ability to sort cells carrying unfixed mutations before they divide and isolate the very cells in which the mutations occurred. This is possible because cell sorting, unlike replica plating, does not require cell division, if the mutational event has occurred in the coding DNA strand. Once these cells divide, if mismatch repair has not occurred, they should give rise to sectored colonies.

Finally, there exist mutant mammalian cell lines with purine

biosynthetic defects which are analagous to the ade1 and ade2 mutations. For example, the Ade D complementation group described by Patterson et al. (8), controls essentially the same biosynthetic reaction as the ade1 gene of yeast. We are therefore suggesting that the FACS approach to genomic DNA alterations in yeast can be extended to more complex organisms such as mammalian cells.

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Table 1. Relative red/white plating efficiencies[a], following sorting of red and white cells from artificial mixtures.

Sorting window	90%-RED/ 10%-WHITE	10%-RED/90% WHITE
White	71.3% W	99.3% W
Red	98.7% R	93.9% R

[a] Efficiencies are given as the percentage of one color colony obtained after plating at least 1000 cells sorted in that color window.

FIGURE LEGENDS

Fig. 1. a) Fluorescence microscopy of yeast strain CBN1-R: fluorescence observed using the standard Zeiss FITC filter set 487709, b) Fluorescence of CBN1-R observed using the Zeiss TRITC filter set 487715. Microscopy was performed using a Zeiss photomicroscope II with a vertical fluorescence illuminator and a Planaplo 40X oil immersion objective. c) Fluorescence emission spectra of trichloracetic acid-insoluble fractions of cytosol preparations of strains S288C and CBN1-R, with excitation at 488 nm. The y-axis represents fluorescence intensity in photon counts. Dotted line; strain CBN1-R, Dashed line; strain S288C, solid line; S288C subtracted from CBN1-R. The cytosol was prepared from yeast strains by two passages through a French Pressure Cell at 16,000 psi, followed by 90 minutes centrifugation at 20,000 rpm in the Beckman 50Ti rotor to pellet unbroken cells and debris. Fluorometry was performed using a Spex Fluorolog fluorometer. Strain CBN1-R was constructed by mating MJL124-11C (α ade1 trp5 ura3 leu2-k met13 lys2 can1 cyh2) obtained from Dr. James Haber's laboratory, with N439-5C (a cdc6 his2 his6 his7 ade1 ade2 ade6 lys7 lys9 leu1 leu2 ura3 trp1 met2 met14 aro7 arg1 arg4 ilv3 asp5 gal1 mal suc) obtained from the Yeast Genetic Stock Center. Strain S288C (α SUC2 mal mel

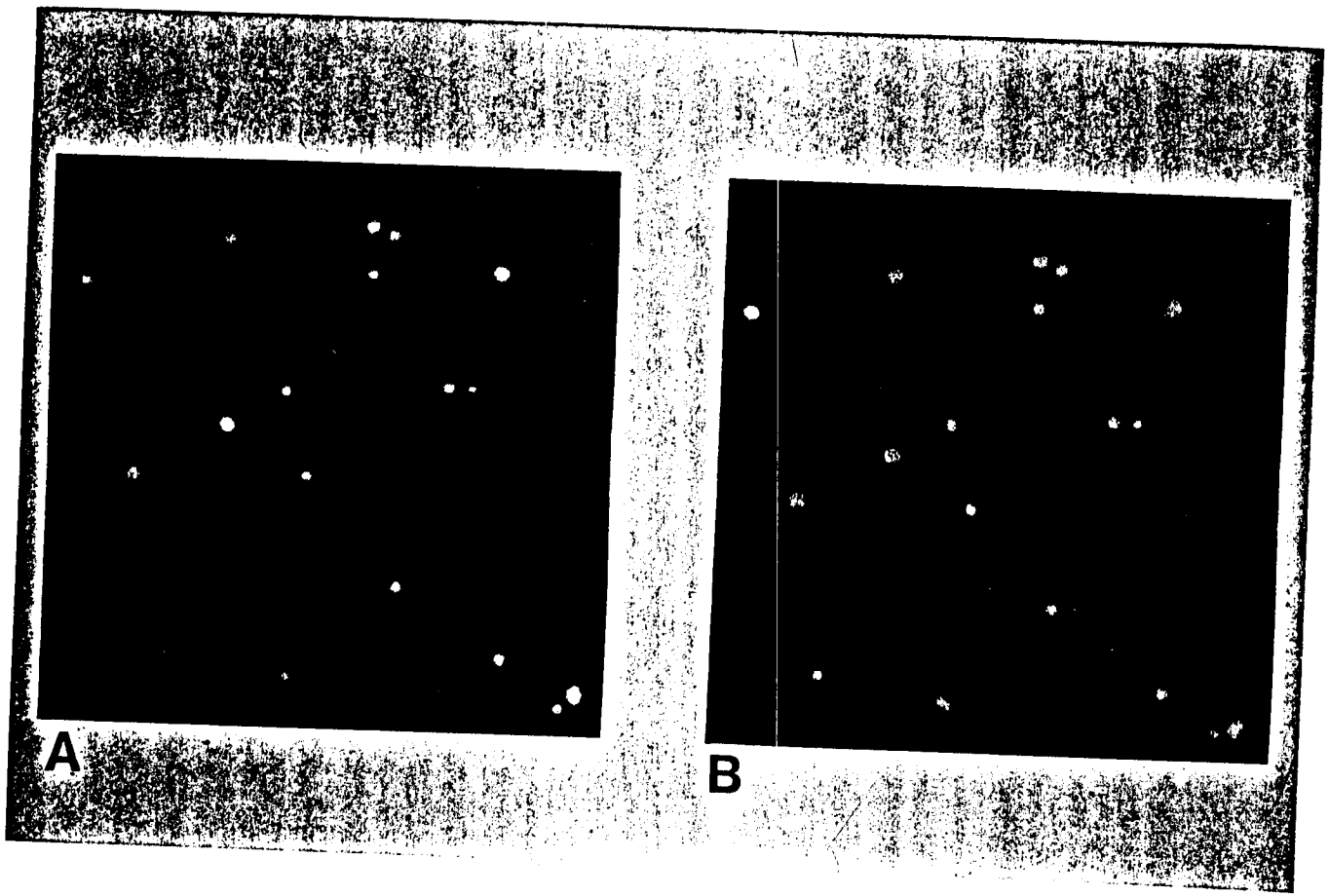
gal2 CUP1 can^{r}), was also obtained from the Yeast Genetic Stock Center. The recipes for media have been described previously (9) Strains were maintained on selective media, or frozen in 50% (v/v) glycerol at -70 C.

Fig. 2 FACS analysis of mixtures of red (strain CBN2-R) and and white (strain CBN2-W) yeast strains. Isometric graphic analysis of fluorescence emission data collected with the FACS 440 cell sorter (Becton Dickinson) with excitation at the 488 nm argon laser line is shown. Fluorescence emission data were collected with two photomultiplier tubes set at right angles, after passing through 530 nm band pass filter and 570 nm long pass filter respectively, and a 560 nm dichroic mirror. Small angle and right angle light scatter data were also collected. Log fluorescence intensity using a 570 nm long pass filter (PMT1) is shown on the F1-axis, log fluorescence intensity using a 530 nm band pass filter (PMT2) is shown on the F2-axis, and cell number is shown on the Z-axis: a) 100% red, b) 100% white, c) 90% red/ 10% white, d) 90% white/ 10% red, e) 80% red/ 20% white, f) 80% white/ 20% red. Strain CBN2-W (cdc6 homozygous) was constructed by mating N439-5C and N361-9A (α cdc6 his4 mal gal SUC), from the Yeast Genetic Stock Center. Strain CBN2-R was a red derivative of strain CBN2-W obtained after induction of chromosomal loss at

36 C. For FACS analysis, cells were grown in complete medium or in complete medium containing low adenine (10 µg/ml), to reduce the chances of feedback inhibition of purine biosynthesis by free adenine.

Fig. 3 Isometric graphic analysis of fluorescence emission data collected from mixtures of red (strain CBN1-R) and white (strain CBN1-W) yeast strains with excitation at 514 nm. Log fluorescence using a 570 nm long pass filter is shown on the F1-axis, log fluorescence using a 530 nm long pass filter is shown on the F2-axis, and cell number is shown on the z-axis: a) 90% red/ 10% white, b) 80% red/ 20% white, c) 80% white/ 20% red, d) 90% white/ 10% red. Strain CBN1-W was a white haploid derivative of CBN1-R obtained by sporulation and selection for recessive canavanine resistance.

Fig. 4 Light scatter analysis of two populations of strain CBN1-R. Log fluorescence using a 530 nm long pass filter is shown on the x-(Fluor 1) axis, and log fluorescence using a 570 nm long pass filter is shown on the y-(Fluor 2) axis in the top panel. Contours were drawn corresponding to various percentages of the total height of the peaks (rising out of the plane of the page). The windows (A and B) in the top panel, corresponding to white and red populations respectively, were analyzed separately for forward (FWD) and right-angle (90) light scatter parameters (shown in the panels marked A and B).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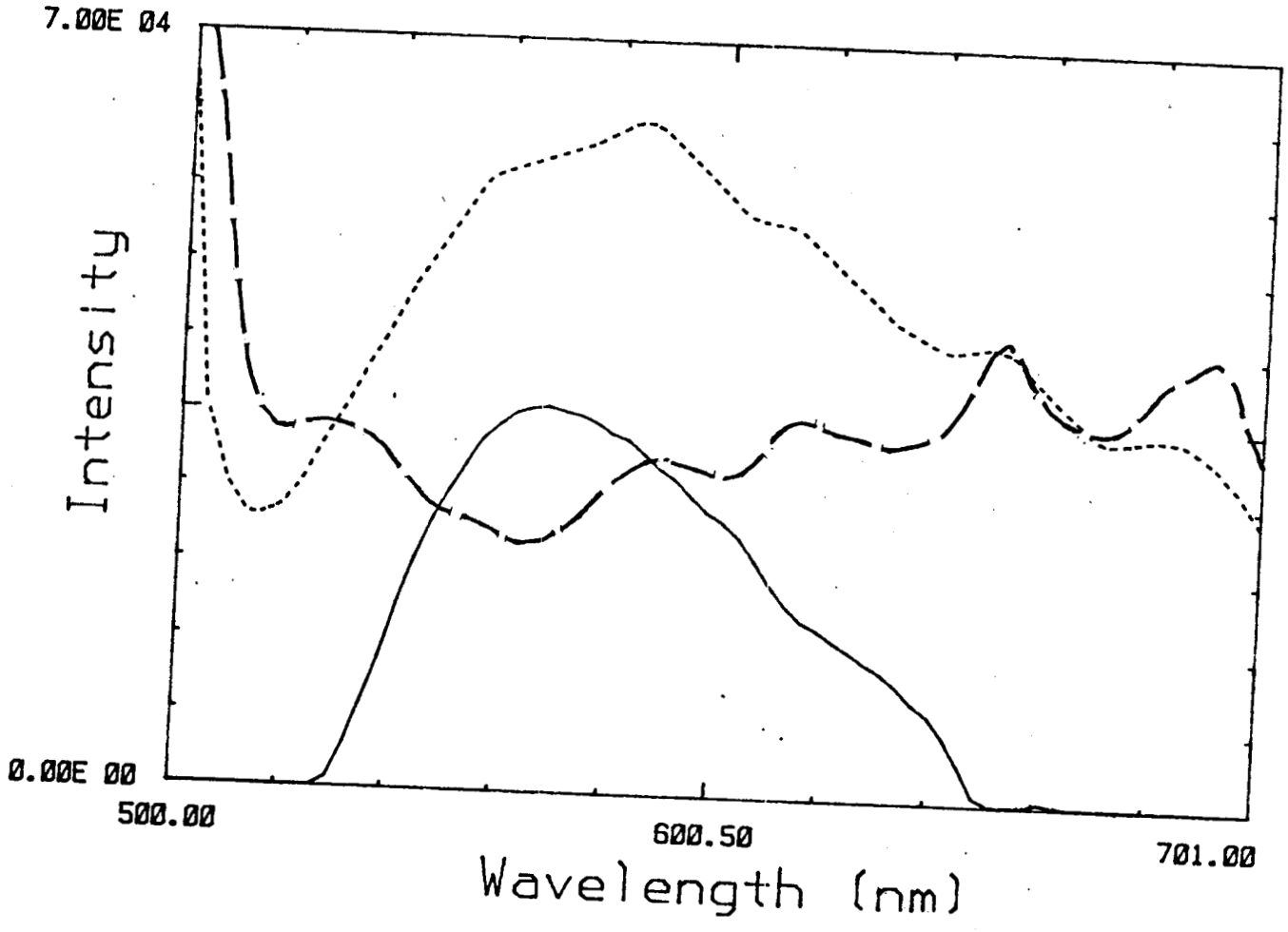
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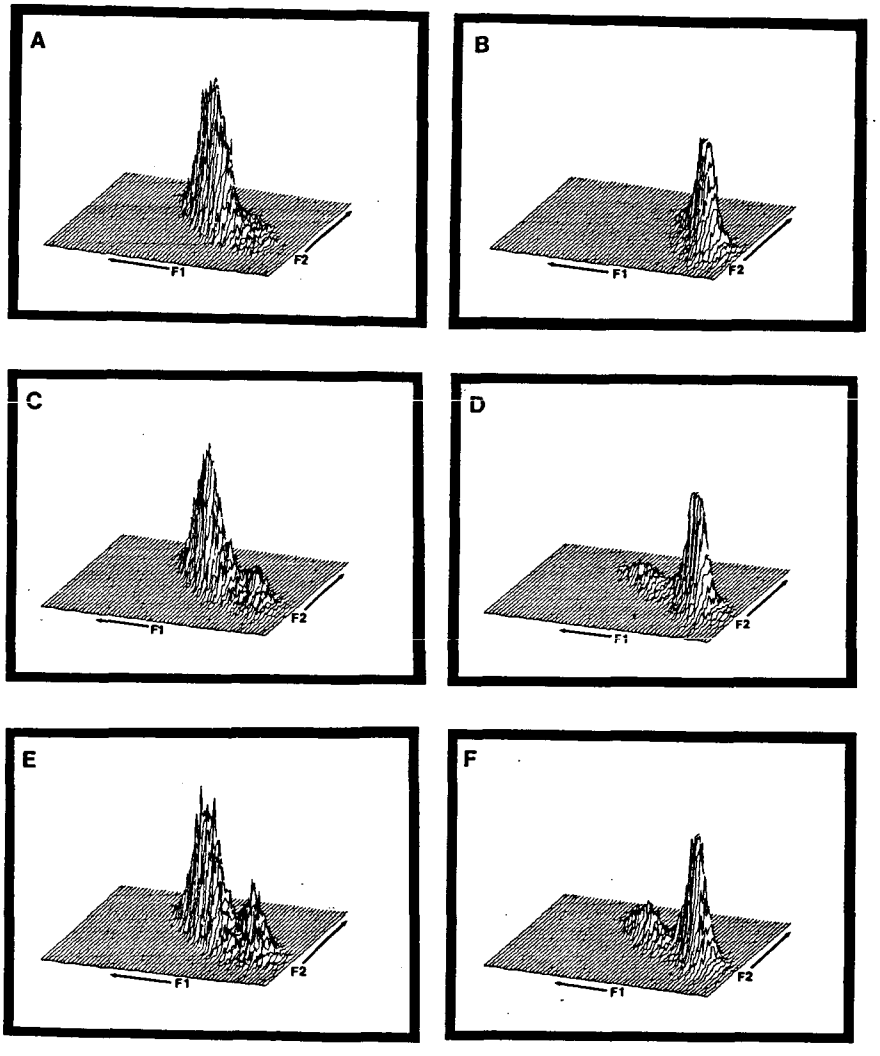
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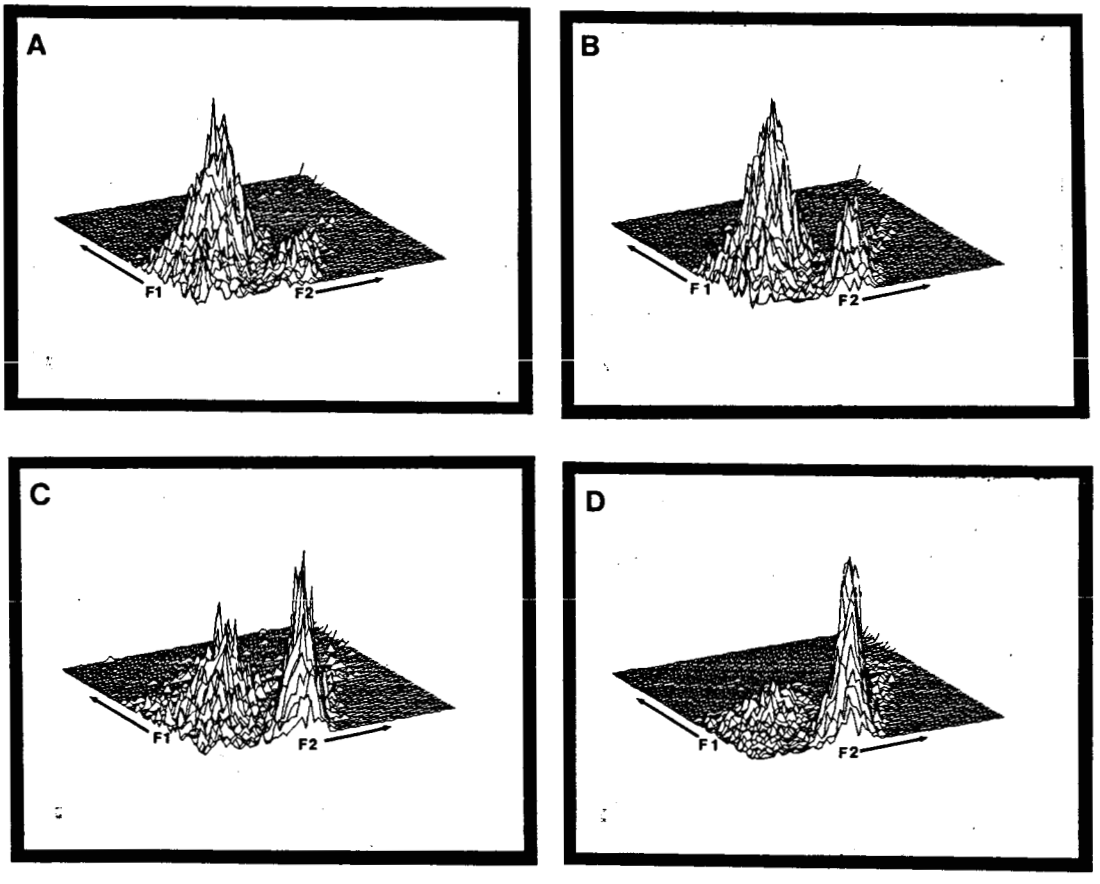
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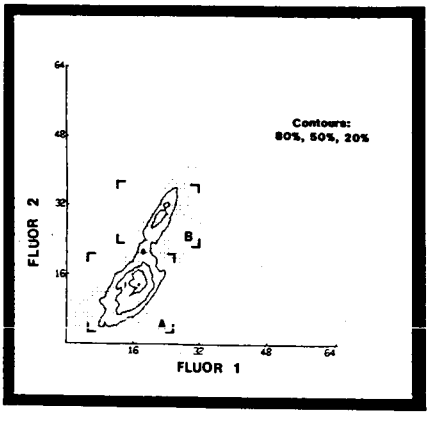
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FLUOR 1
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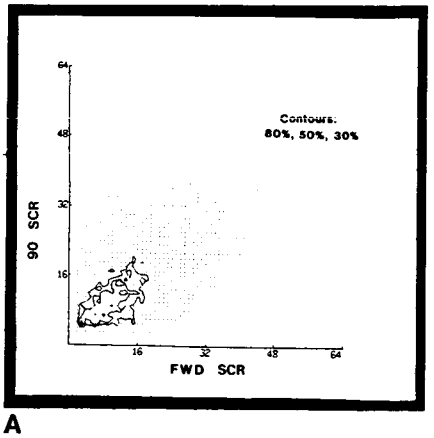
48
32
16
A
B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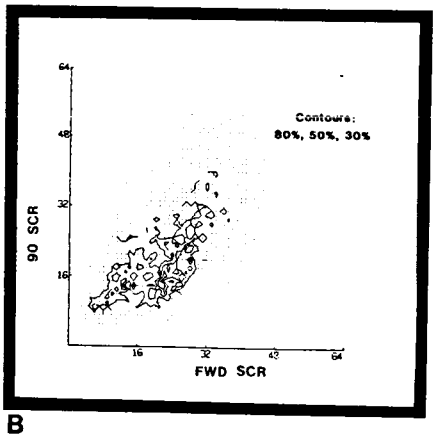
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